

New records from Lithuania of fungi alien to Europe

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ABSTRACT — First records from Lithuania of the ascomycete *Ophiostoma novo-ulmi* and basidiomycetes *Clathrus archeri*, *Leucocoprinus cepistipes*, and *Stropharia rugosoannulata* are presented. All four species are alien to Europe and two, *C. archeri* and *S. rugosoannulata*, have never been recorded in the eastern part of the Baltic Sea region before. Also, a reassessment of the status in Lithuania of the alien pathogen *Hymenoscyphus fraxineus* and its close relative, the non-pathogenic *H. albidus* indigenous to Europe, indicates that only *H. fraxineus* occurs in Lithuania. Descriptions of the examined fungi are presented, and remarks on their habitats and distribution are provided.

KEY WORDS — anthropogenic habitats, distribution, invasive species, non-native fungi, saprobes

Introduction

Precise data on diversity and distribution of fungi, their geographical ranges, and habitat requirements are often lacking even for comparatively well-studied regions. Fragmentary baseline knowledge of fungal taxonomy, ecology, and geographical distribution is probably one reason why research on alien organisms and their effect on ecosystems has so far paid insufficient attention to fungi (Pyšek et al. 2008), with the exception of some invasive pathogenic species that cause conspicuous damage to vegetation or wildlife (Desprez-Loustau et al. 2010). Until recently, the spread of alien mycorrhizal and saprobic macromycetes in particular was treated as a mycological curiosity, although

lately the economical and ecological importance of alien non-pathogenic fungi has been acknowledged (Richardson et al. 2000, Desprez-Loustau et al. 2007, Vellinga et al. 2009, Nuñez & Dickie 2014). Nonetheless, the general knowledge of geographical distribution of alien fungi is far from complete (Kreisel 2006, Desprez-Loustau et al. 2010, Santini et al. 2013) as information on new localities of already established alien fungi and on new arrivals continuously accumulates. Moreover, accurate identification is often an issue for reports on the occurrence of alien macromycetes — e.g., *Amanita phalloides* (Fr.) Link in North America; Pringle & Vellinga 2006 — and plant pathogens — e.g., *Hymenoscyphus fraxineus* (Baral & Bemmam 2014, Gross et al. 2014) and subspecies and hybrids of *Ophiostoma novo-ulmi* (Brasier 1991, Brasier & Buck 2001, Brasier & Kirk 2001) — in Europe.

In Lithuania, the first records of fungi (including aliens) appeared almost 200 years ago; since then the number of non-native species records has continuously increased, although so far there is still no comprehensive list of such fungi in the country. Here we report and discuss the first cases of occurrence of four fungal species that are alien in Europe, and revise the status of an invasive ash dieback pathogen, *Hymenoscyphus fraxineus*, and its close relative indigenous saprobe, *H. albidus* (Gillet) W. Phillips, in Lithuania.

Materials & methods

EXAMINATION OF FUNGAL FRUIT-BODIES. Fresh basidiomata of *Leucocoprinus cepistipes* and *Stropharia rugosoannulata* and dried basidiomata of *Clathrus archeri* were studied. Additionally, color photographs of *C. archeri* in situ were examined. Microscopical features were examined under a Nikon Eclipse Ci-S light microscope. Squashed preparations were mounted in water and in 5% KOH solution. All measurements were made in water. Herbarium specimens previously identified as *Hymenoscyphus albidus* were examined for presence of simple septa or croziers at the bases of asci. Squashed sections or fragments of ascomata were mounted in 3% KOH + Lugol's iodine solution or Congo Red and studied under an Olympus BX51 light microscope. All descriptions are based on Lithuanian material.

MOLECULAR IDENTIFICATION OF *OPHIOSTOMA NOVO-ULMI* ISOLATES. A total of 49 *O. novo-ulmi* isolates were recovered from wood of wilting branches of 20 *Ulmus glabra* and 29 *U. minor* trees growing in various parts of Lithuania (for details of the localities see Norkutė 2013). In the laboratory, the fungi were isolated and cultured according to Brasier (1981) and Bakys et al. (2009). All 49 isolates that were morphologically similar to *O. novo-ulmi* based on cultural descriptions by Brasier (1991) and Kirisits & Konrad (2004) were assigned for DNA extraction and grown for two weeks on liquid Hagem media in static cultures at room temperature. Protocols for DNA extraction and PCR amplifications followed Kären et al. (1997), and the ribosomal ITS region was sequenced by Macrogen Inc. (Seoul, Korea) using two primers (ITS1F and ITS4; White et al. 1990). All obtained sequences were identical and identified via a NCBI BLAST

database (www.ncbi.nlm.nih.gov) search as *O. novo-ulmi*. The representative sequence of the Lithuanian *O. novo-ulmi* (LPM ON3; for details see below) has been deposited at GenBank (accession no. KJ677112).

PCR-RFLP of the *cerato-ulmin* (*cu*) and colony type (*COL1*) genes was used to identify *O. novo-ulmi* subspecies and hybrids following the protocol developed by Konrad et al. (2002): DNA of all 49 isolates was amplified using DreamTaq™ Green DNA polymerase (Fermentas, Vilnius, Lithuania) following the manufacturer's instructions with a different annealing temperature for each primer (68°C for *cu*, 58°C for *COL1*). The restriction enzymes BfaI and HphI (Fermentas, Vilnius, Lithuania) were used in the RFLP process according to manufacturer instructions and following recommendations by Konrad et al. (2002). Restriction products were visualized in 2% agarose gel (90 min at 120 V in sodium borate (SB) buffer; Fig. 1). Assignment of the screened *O. novo-ulmi* isolates to subspecies or hybrids followed Konrad et al. (2002).

SPECIMEN ACCESSIBILITY. All specimens cited below are deposited in fungal collections of either the Herbarium of the Institute of Botany of Nature Research Centre, Vilnius, Lithuania (BILAS) or the Herbarium of Vilnius University, Vilnius, Lithuania (WI). Isolates of *O. novo-ulmi* are stored in the culture collection of the Laboratory of Phytopathogenic Microorganisms at the Institute of Botany of Nature Research Centre, Vilnius, Lithuania (LPM).

Nomenclature follows MycoBank Fungal Databases (<http://www.mycobank.org>).

Clathrus archeri (Berk.) Dring, Kew Bull. 35: 29. 1980.

Immature basidiomata globose to ovoid, dirty whitish, growing solitary or gregarious. Mature basidiomata with 4–6 (most commonly five) receptaculum arms initially fused at the tips, later splitting and spreading outwards; arms tapered to apices, bright red on the inner (upper) surface and pale red elsewhere, finely reticulately pitted, with dark green gleba. Receptaculum of rounded cells, 40–50 µm diam. Gleba emitting a strong carrion-like odor (persistent even in dried material). Stem with the base encased in a whitish volva. Basidiospores cylindric-ellipsoid, hyaline, greenish in mass, smooth-walled, 4.5–6 × 2–2.5 µm. Basidia not observed.

SPECIMEN EXAMINED: LITHUANIA, KRETINGA DISTR., northern edge of Kartena village, 55°56'N 21°28'E, 1 Sept 2011, E. Slušnytė (BILAS 49286).

HABITAT IN LITHUANIA: On soil on a semi-open slope, covered with shrubs and in herbaceous vegetation between planted young *Picea abies* and *Pinus sylvestris*.

NOTES: Originally described from Tasmania, and indigenous in Australia and New Zealand, *Clathrus archeri* has spread to Europe, North America, and Asia as an alien species. Since it was first recorded from France in 1914, the fungus has spread widely across western and central Europe (Michael et al. 1986, Pegler et al. 1995, Hansen & Knudsen 1997, Birsan et al. 2014). The records of *C. archeri* closest to Lithuania are from Poland, mostly from the southern part where it has a status of a spreading species, although the reports from

northern Poland are few and at the present time confined to the coastal areas with a milder climate (Miądlukowska 1995, Halama et al. 2010, Szczepkowski & Obidziński 2012). In Lithuania, the fungus was found in the west, also characterized by a milder climate influenced by the Baltic Sea. Kreisel (2006) noted that elsewhere in Europe, *C. archeri* continues to spread northwards, where it has already become established in southern Sweden (M. Jeppson, personal communication). Our present record is the first for the eastern Baltic Sea region.

Hymenoscyphus fraxineus (T. Kowalski) Baral, Queloz & Hosoya, IMA Fungus 5: 79. 2014.

Ascomata developing on blackened (stromatized) petioles of fallen previous year's leaves of *Fraxinus excelsior*, gregarious, superficial, stipitate, cup-shaped, 1–4 mm diam. Hymenium shallowly concave or plane, whitish or cream to ochraceous. Margin even or sinuate, hairless. Receptacle concolorous with the hymenium, somewhat striate. Stipe obconical to cylindrical, concolorous with the receptacle, blackened at the base, ≤ 1.2 mm long. Exterior covered by short hyphal outgrowths, especially on stipe. Ectal excipulum a textura prismatica (-porrecta), cells with slightly gelatinized walls. Medullary excipulum a hyaline textura intricata. Asci arising from croziers, cylindric-clavate, 8-spored, $85\text{--}120 \times 8\text{--}14$ μm , apical pore blue in Lugol's solution. Ascospores cylindrical to cylindric-clavate, straight or slightly curved, often inequilateral, usually scutuloid, with internal droplets, aseptate or occasionally 1-septate, $16\text{--}24 \times 3\text{--}5$ μm . Paraphyses cylindrical, apically unthickened or slightly inflated ≤ 3 μm .

SPECIMENS EXAMINED: LITHUANIA, KĖDAINIAI DISTR., Stebuliai forest, $55^{\circ}19'N$ $24^{\circ}07'E$, 21 Sept 1999, E. Kutorga (BILAS 34422), 29 Aug 2000, E. Kutorga (WI 7344); Vilainiai forest, $55^{\circ}18'N$ $24^{\circ}02'E$, 21 Sept 1999, E. Kutorga (BILAS 34426), 30 Aug 2000, E. Kutorga (WI 7345); Pašiliai forest, $55^{\circ}14'N$ $23^{\circ}58'E$, 1 Jun 2000, E. Kutorga (WI 7341); Lančiūnava forest, $55^{\circ}20'N$ $24^{\circ}12'E$, 29 Aug 2000, E. Kutorga (WI 7342); Berankiškis forest, $55^{\circ}14'N$ $24^{\circ}08'E$, 29 Aug 2000, E. Kutorga (WI 7343), 3 Oct 2001, E. Kutorga (BILAS 25151), 2 Oct 2002, E. Kutorga (BILAS 34425); Juodkiškiai forest, $55^{\circ}16'N$ $24^{\circ}02'E$, 30 Aug 2000, E. Kutorga (WI 7346); Šilainėliai forest, $55^{\circ}14'N$ $24^{\circ}02'E$, 3 Oct 2002, E. Kutorga (BILAS 33994); ŠIRVINTOS DISTR., Širvintos forest, $55^{\circ}03'N$ $24^{\circ}58'E$, 10 Sept 1997, E. Kutorga (WI 7409); UKMERGĖ DISTR., Taujėnai forest, $55^{\circ}26'N$ $24^{\circ}39'E$, 11 Sept 1997, E. Kutorga (WI 7410); VILNIUS CITY, Verkiai forest, $54^{\circ}45'N$ $25^{\circ}17'E$, 25 Jun 2001, A. Kazlauskienė (WI 4714).

HABITAT IN LITHUANIA: Ash dieback symptoms occur in most habitats where *F. excelsior* grows. The ascomata of *H. fraxineus* were registered in leaf litter in different forests, although most commonly in mixed deciduous stands. Ascomata were also recorded from parks and nurseries under diseased trees, but these records were not confirmed microscopically.

NOTES: The causal agent of ash dieback, pathogenic *H. fraxineus*, is an alien invasive ascomycete in Europe that is replacing the closely related saprobic

indigenous species, *H. albidus* (Queloz et al. 2011, 2012; McKinney et al. 2012; Baral et al. 2014; Gross et al. 2014). *Hymenoscyphus fraxineus* is well known from the characteristic disease symptoms (Gross et al. 2014) and molecular data obtained from both isolated fungal cultures and symptomatic ash tissues. In Lithuania, crown dieback of *F. excelsior* was first observed in 1996 and by 2001 it had already become widespread (Juodvalkis & Vasiliauskas 2002). The disease has devastated ash stands all over the country and currently is in its chronic phase (Pliūra et al. 2011, Bakys 2013, Lygis et al. 2014). Initially, its primary causal agent was not properly diagnosed (Lygis et al. 2005, Vasiliauskas et al. 2006). The fungus was correctly identified only in 2008 when DNA sequence of a fungus (labelled as *Hymenoscyphus* sp. 970 by Lygis et al. in 2005) isolated in 2001 from a root collar of declining *F. excelsior* was assigned to *H. fraxineus* (GenBank AY787704, as *Chalara fraxinea*).

Since 1997, stromatized *F. excelsior* leaf petioles with numerous ascomata of *Hymenoscyphus* sp. collected in Lithuania have been deposited in herbaria BILAS and WI. The ascomata were first assigned to the saprobic *H. albidus* (Treigienė et al. 2010). Recently, Zhao et al. (2012) and Baral & Bemmam (2014) observed subtle microscopic differences in ascomatal structures between closely related *H. albidus* and *H. fraxineus* and underlined the importance of combining detailed morphological studies with molecular analysis in the *H. albidus* / *H. fraxineus* species complex. They reported that the presence or absence of croziers at the ascus base could distinguish *H. albidus* from *H. fraxineus*, differences that are strictly correlated with molecular characteristics. Therefore, we re-examined all herbarium specimens microscopically in order to clarify the status of the two *Hymenoscyphus* species in Lithuania. As the asci of all studied specimens had croziers, they were assigned to *H. fraxineus*. This suggests that there is no official proof of previous or current occurrence of *H. albidus* in Lithuania. It is worth mentioning that the Lithuanian collections of *H. fraxineus* specimens with ascomata (collected between 1997–2002) represent one of the earliest herbarium records of this fungus in Europe.

Leucocoprinus cepistipes (Sowerby) Pat., J. Bot. 3: 336. 1889.

Basidiomata growing in clusters. Pileus 2–6 cm diam, conic-campanulate when young, later convex to plano-convex, with an obtuse umbo. Surface dry, white to cream-colored, fibrillose, soon developing small white to cream-colored scales. Context white, soft, very thin. Lamellae free, narrow, white, becoming pallid to pale buff, edges white-floccose. Stipe 3–10 × 0.3–0.6 cm, cylindrical, hollow; base swollen, stipe surface above the annulus white and smooth, below – finely white-floccose, turning brownish when rubbed. Annulus fibrillose-membranous, white, with a somewhat crenate margin. Odor and taste mild,

not distinctive. Basidiospores ellipsoid to ovoid, smooth, hyaline, with a small germ pore, $6\text{--}11 \times 5\text{--}7 \mu\text{m}$. Basidia clavate, with 4 sterigmata, without basal clamp, $22\text{--}35 \times 10\text{--}12 \mu\text{m}$. Cheilocystidia clavate, sometimes rostrate, $25\text{--}60 \times 8\text{--}15 \mu\text{m}$. Pleurocystidia absent.

SPECIMENS EXAMINED: LITHUANIA, VILNIUS CITY, Valakupiai, $54^{\circ}44'N$ $25^{\circ}19'E$, 10 Jun 2005, V. Urbonas (BILAS 30747); Fabijoniškės, $54^{\circ}44'N$ $25^{\circ}15'E$, 28 May 2009, V. Kleizaitė (WI 7278).

HABITAT IN LITHUANIA: on soil in a greenhouse and in a flowerpot in a private apartment.

NOTES: *Leucocoprinus cepistipes* is of tropical origin, native to Asia and Africa; it has been introduced to Europe with a plant material and is apparently spreading (Vellinga 2001). In central and northern Europe it grows almost exclusively indoors (Pidlich-Aigner et al. 2002, Knudsen & Vesterholt 2008, Gierczyk et al. 2011); however in western and southern Europe the fungus has been found also outdoors on humus-rich soil, woodchips, compost or manure. In northern Poland, *L. cepistipes* was once recorded outdoors, growing on a horticultural waste dump (Gierczyk et al. 2011); however, there are no data of its overwintering. In Latvia (Riga city), the fungus was found in a lawn strip between a street and a sidewalk (Daniele & Meiere 2011; I. Daniele, personal communication). In Lithuania, *L. cepistipes* has been found exclusively indoors.

Ophiostoma novo-ulmi Brasier, Mycopathologia 115: 155. 1991.

No sexual and asexual fruiting structures of *O. novo-ulmi* were observed in Lithuania. Disease symptoms were in accordance with those provided by Anonymous (2009). Characteristics of the cultures coincided with those provided by Brasier (1991).

MATERIAL EXAMINED: (for geographical coordinates see Norkutė 2013).

subsp. *novo-ulmi*: LITHUANIA, BIRŽAI DISTR., Karajimiškis, from *Ulmus glabra*, May 2011 (LPM ON13); KAUNAS DISTR., Raudondvaris, from *U. glabra*, Nov 2011 (LPM ON43); KĖDAINIAI DISTR., Aristava, from *U. minor*, Jun 2011 (LPM ON14, ON15, ON18, ON19, ON21–ON34, ON36–ON39, ON42); PASVALYS DISTR., Gulbinėnai, from *U. glabra*, May 2011 (LPM ON10); Paberliai, from *U. glabra*, May 2011 (LPM ON11, ON12); PLUNGĖ DISTR., Kuliai, from *U. glabra*, Jun 2011 (LPM ON48–ON51); ROKIŠKIS DISTR., Dusetos, from *U. glabra*, Jun 2011 (LPM ON44–ON47).

subsp. *novo-ulmi* $\times$ subsp. *americana* hybrid: LITHUANIA, KĖDAINIAI DISTR., Aristava, from *Ulmus minor*, Jun 2011 (LPM ON16, ON17, ON20, ON35, ON40, ON41); PLUNGĖ DISTR., Kuliai, from *U. glabra*, Jun 2011 (LPM ON52, ON53); VILNIUS CITY, Verkių park, from *U. glabra*, 12 Jul 2010 (LPM ON3; GenBank KJ677112); VILNIUS DISTR., Verkšionys, from *U. glabra*, 19 Jul 2010 (LPM ON5, ON6); Dūkštos, from *U. glabra*, 19 Jul 2010 (LPM ON7, ON8).

HABITAT IN LITHUANIA: According to information provided by the Department of Forest Sanitary Protection at Lithuanian State Forest Service (personal communication), Dutch elm disease symptoms occur in most habitats where elms (*Ulmus* spp.) grow.

NOTES: *Ophiostoma ulmi* (Buisman) Nannf. s. str. caused the original Dutch elm disease epidemic in Europe and North America in the mid-1900's (Gibbs 1978). *Ophiostoma novo-ulmi*, a more aggressive pathogen of elms, largely replaced *O. ulmi* during the second half of the 20th century (Brasier 1991). Seemingly, *O. ulmi* s. str. is already absent in most of the European countries as no recent data on occurrence of this species is available. *Ophiostoma novo-ulmi* has two subspecies – *O. novo-ulmi* Brasier subsp. *novo-ulmi* (Eurasian, EAN) and *O. novo-ulmi* subsp. *americana* Brasier & S.A. Kirk (North American, NAN) (Brasier & Kirk 2001). The species is invasive in Europe: NAN was introduced from North America in 1960's and EAN spread across Europe from east to west at about the same time (Brasier & Gibbs 1973, Brasier 1990, 1996).

In Lithuania, the outbreak of Dutch elm disease and the causative agent *O. ulmi* s. str. were first recorded in mid-20th century (Žuklys 1958), but afterwards this pathogenic ascomycete was not observed. Our molecular identification of fungal isolates from wood of wilting elm branches collected in 2010–2011 from different regions of Lithuania has demonstrated that all isolates represent *O. novo-ulmi*. FIG. 1 shows the RFLP banding patterns of *CU* and *COL1* genes from seven Lithuanian *O. novo-ulmi* isolates digested with restriction enzymes *HphI* and *BfaI*; according to the *CU* and *COL1* gene fragment sizes (see Konrad et al. 2002), isolates LPM ON47–ON51 (lanes 1–5) correspond with subspecies EAN, while LPM ON52 and ON53 (lanes 6 and 7) correspond with EAN ×

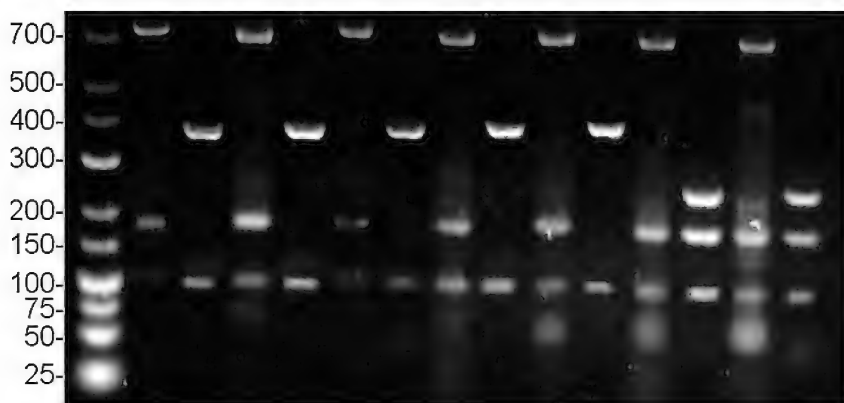


FIG. 1. RFLP banding patterns of cerato-ulmin gene *CU* from *Ophiostoma novo-ulmi* digested with restriction enzyme *HphI* (lane numbers labelled "c") and colony type gene *col1* digested with restriction enzyme *BfaI* (lane numbers labelled "l") after separation on a 2% agarose gel. Lane numbers represent seven *O. novo-ulmi* isolates: 1 = ON47; 2 = ON48; 3 = ON49; 4 = ON50; 5 = ON51; 6 = ON52; 7 = ON53 (see also Norkutė 2013). M = DNA marker, Thermo Scientific GeneRuler Low Range DNA Ladder (Thermo Fisher Scientific Inc.).

NAN hybrid. On the basis of similar RFLP patterns from all 49 Lithuanian *O. novo-ulmi* isolates, 36 were identified as EAN and 13 as EAN × NAN hybrid (Norkutė 2013). Our results suggest that *O. novo-ulmi* has replaced *O. ulmi* s. str. in Lithuania.

Stropharia rugosoannulata Farl. ex Murrill, Mycologia 14: 139. 1922.

Basidiomata numerous, growing solitary or gregarious. Pileus 5–15 cm diam, convex to broadly convex, surface dry, smooth, wine red to reddish brown, or pale brownish, sometimes cracked in old age (both color types were present in the Lithuanian sample); the margin with partial veil remnants. Context white, not changing color upon exposure. Lamellae broadly adnate, gray when young, later gray blue, black violet, close. Stipe 7–15 × 3 cm, central, cylindrical to slightly clavate, brittle; surface above the annulus white and finely striate, surface below with longitudinal fibrils, which are whitish at first and later pale yellowish on a whitish background; base with white mycelial threads. Annulus compact, pendent, white; surface finely striate on its upper side; margin splitting causing annulus to become stellate. Odor pleasant, taste mild. Basidiospores brownish-violet, ellipsoid to ovoid, smooth, thick walled, with a germ pore, 10–13 × 7.5–9 µm. Basidia clavate, with 4 sterigmata, without basal clamp, 26–33 × 8–9.5 µm. Cheilocystidia ventricose, clavate or lageniform, with apical protrusion, not changing color in KOH, 30–45 × 11–22 µm. Pleurocystidia fusiform-ventricose, some also with contents not changing color in KOH, 28–50 × 10–13 µm. Pileipellis periclinal, composed of brown-pigmented, 5–13 µm thick hyphae; some septa with clamps.

SPECIMEN EXAMINED: LITHUANIA, VILNIUS DISTR., Gineitiškės, 54°44'N 25°11'E, 9 Oct 2011, V. Būda (BILAS 50367).

HABITAT IN LITHUANIA: on wood chips in a garden among shrubs of *Ribes* spp.

NOTES: The fungus has apparently been introduced to Lithuania with imported gardening woodchips, a substrate known as a rich source of alien macromycete species (Shaw & Kibby 2001). *Stropharia rugosoannulata* is a widely cultivated mushroom (although not in Lithuania) that has also recently been observed in natural and semi-natural environments elsewhere in Europe (Breitenbach & Kränzlin 1995, Knudsen & Vesterholt 2008). At least in central Europe, the species is likely to be ephemeral as it usually disappears with deterioration of its substrate (Voglmayr & Krisai-Greilhuber 2002). This hypothesis is supported by Shaw et al. (2004), who state that substrates like woodchips made of ornamental trees can sustain diverse albeit short-lived fungal communities.

This is the first record of *S. rugosoannulata* in the eastern Baltic region.

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